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Current Opinion in Virology

Seeing and touching adenovirus: complementary approaches for understanding assembly and disassembly of a complex virion

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Understanding adenovirus assembly and disassembly poses many challenges due to the virion complexity. A distinctive feature of adenoviruses is the large amount of virus-encoded proteins packed together with the dsDNA genome. Cryo-electron microscopy (cryo-EM) structures are broadening our understanding of capsid variability along evolution, but little is known about the organization of the non-icosahedral nucleoproteic core and its influence in adenovirus function. Atomic force microscopy (AFM) probes the biomechanics of virus particles, while simultaneously inducing and monitoring their disassembly in real time. Synergistic combination of AFM with EM shows that core proteins play unexpected key roles in maturation and entry, and uncoating dynamics are finely tuned to ensure genome release at the appropriate time and place for successful infection.

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Current Opinion in Virology 2022, **52**:112–122

This review comes from a themed issue on **Virus structure and expression**

Edited by **José R Castón** and **Adam Zlotnic**

For complete overview about the section, refer Virus structure & Expression (2022)

Available online 11th December 2021

https://doi.org/10.1016/j.coviro.2021.11.006

Adenoviruses are pathogens with non-negligible clinical relevance, particularly (but not only) in immunosuppressed individuals, and so far there is no efficient treatment for adenovirus-caused infections [1,2]. Additionally, recombinant adenoviruses have long been used as experimental vehicles for therapeutic purposes, with promising outcomes in antitumoral applications [3] and actual widespread use as vaccine vectors [4].

The adenovirus virion weights ~150 × 10^6 Da and is formed by more than 10 different proteins plus the viral genome, a linear dsDNA molecule ranging from 26 to 48 kbp across the *Adenoviridae* family [5–8]. This size and complexity hindered high resolution determination of the adenovirus architecture, even after a titanic effort yielded X-ray diffracting crystals [5,9–11]. At the dawn of the resolution revolution, cryo-EM finally provided the structure of human adenovirus type 5 (HAdV-C5), the family prototype [12,13].

How interactions between all the virion components are orchestrated to produce a correctly assembled, infectious adenovirus particle is poorly understood. Likewise, knowledge is lacking on capsid stability modulation, control and dynamics of uncoating upon cell entry. Deciphering the structural aspects of the infectious cycle would provide essential information to tailor vectors for different therapeutic applications, or to devise drugs to treat adenovirus infections. Although in the latest years there has been an increase in discovery of molecules inhibiting adenovirus infections [14], most of these interfere with the metabolism of viral nucleic acids, not with particle assembly or disassembly. Exceptions are protease inhibitors, and a newly reported compound that seems to preclude endosome escape — however, its exact mechanism of action is not known [15]. No specific anti-adenovirus compounds have reached the clinic so far. Nucleic acid metabolism depends on similar processes for the virus and the host, posing the problem of cytotoxicity for the potential antiviral candidates. Interactions leading to capsid assembly and disassembly are expected to be virus-specific, which provides an interesting alternative for antiviral development. Molecules hindering conformational changes required for picornavirus uncoating; maturation or uncoating inhibitors binding HIV Gag; compounds deregulating the hepatitis B virus assembly pathway; and synthetic peptides competing with a stabilizing minor coat protein for capsid incorporation in Kaposi's sarcoma-associated herpesvirus, have all shown promise as antivirals [16–18].

The importance of understanding physical properties of viral capsids has lately been recognized [19,20]. Characterizing viral particle mechanics should help understand how they fulfill the conflicting stability needs along the infectious cycle. Additionally, mechanical stress may play significant roles not only as a deleterious factor, but also as a determinant of infectious cycle processes such as cell entry and genome delivery [21,22]. AFM allows investigating the physical properties of virus particles adsorbed

on a surface in liquid milieu, by using a sharp stylus located at the end of a microcantilever [23,24]. AFM can manipulate individual capsids in real time to obtain nanoscale topographical maps, probe their nanomechanics, and alter their structure in a controlled fashion to learn about the uncoating process and the influence of changing environments [21,25,26].

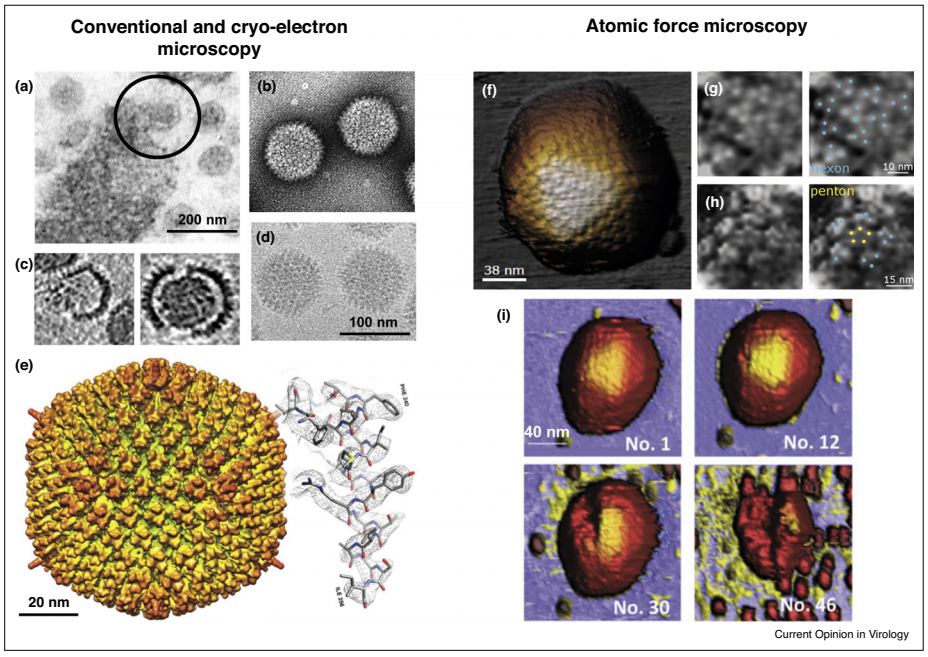
Conventional EM has long been used to follow virus-related modifications in infected cells. Cryo-EM single particle averaging provides highly detailed information on the architecture of the icosahedral shell, while AFM manipulation of individual particles reveals their mechanical properties and disassembly dynamics (Figure 1). Here we discuss how the combination of these techniques is providing substantial new insights on adenovirus architecture, assembly, stability and uncoating.

Architecture of the adenovirus capsid

Several new adenovirus structures have lately been produced by cryo-EM, including HAdV-C5 at improved resolution (3.2 Å versus 3.5 Å in 2010) [12,27] and the first high resolution structure of an adenovirus with a non-mammalian host, lizard adenovirus LAdV-2 [28••]. Structures of human adenoviruses HAdV-D26, causing conjunctivitis, and HAdV-F41, causing gastroenteritis, were interpreted in terms of their different tropisms [29,30•,31•]. The structure of the chimpanzee adenovirus used as vector for one of the COVID-19 vaccines was solved to search the source of blood clot disorders appearing in a few vaccine recipients [32].

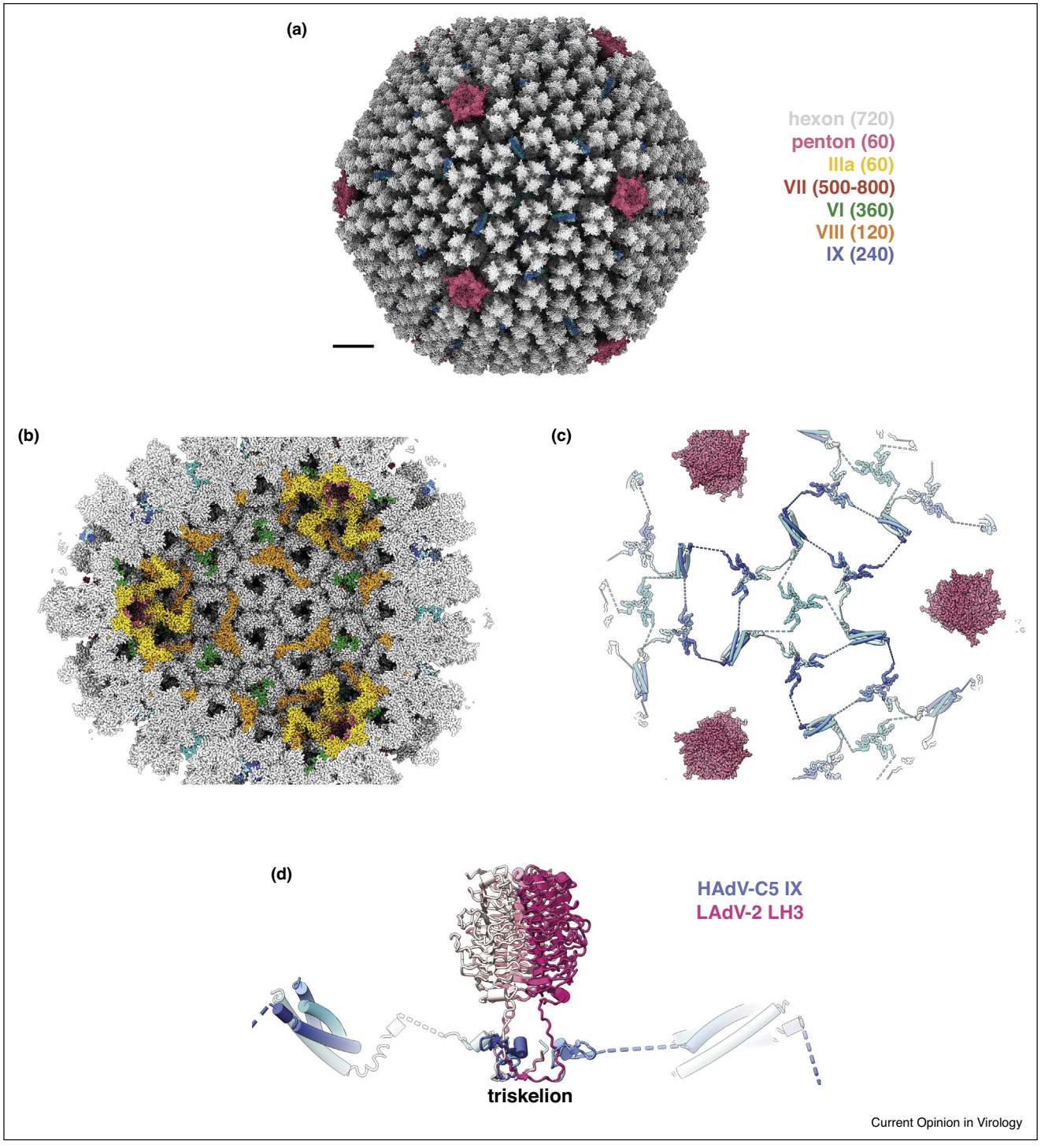
Adenoviruses have non-enveloped, *pseudo T* = 25 icosahedral capsids with diameter between 88 (facet–facet) and 95 (vertex–vertex) nm (Figure 2a). Their major capsid protein (hexon) folds as a double jelly roll perpendicular

Figure 1



Example images illustrating different kinds of information provided by electron microscopy and atomic force microscopy.
(a) Conventional electron microscopy: detail of a thin section obtained from freeze-substituted HEK293 cells infected with a HAdV-C5 delayed packaging mutant. A partially formed capsid engulfing DNA in the adenovirus peripheral replication zone is indicated with a circle. Modified from Ref. [51]. **(b)** Two HAdV-C5 particles imaged by negative staining. **(c)** Details of cryo-electron tomograms showing disassembly intermediates of mature (left) and immature (right) adenovirus particles. Notice that after particle disruption, the immature core remains condensed forming a sphere that stays attached to capsid fragments. Modified from Ref. [67]. **(d)** Two HAdV-C5 particles imaged by cryo-electron microscopy. The scale bar in (d) applies also to (b) and (c). **(e)** A HAdV-C5 3D map obtained from cryo-electron microscopy single particle averaging, with a detail of a molecular model fit onto high resolution density for LAdV-2 at the right. **(f)** 3D rendered topographical image of a HAdV-C5 particle obtained with the AFM in liquid. **(g)** and **(h)** show high pass filtered topographies of the facet center and the vertex region, respectively, revealing the triangular towers of the hexons and the pentameric structure of the penton. On the right hand side panels, the lobes of the penton and hexons are highlighted with yellow and blue dots, respectively. Panels (f)–(g) reproduced from Ref. [81] and kindly provided by A. Ortega-Esteban. **(i)** Four images taken during a mechanical fatigue AFM assay (at 100 pN) showing the sequential disassembly of a HAdV-C5 particle. Top-left: intact particle. Top-right: the same particle with the three pentons bordering the topmost icosahedral facet lost. Bottom-left: the same particle after the shell has cracked and its contents start being released. Bottom-right: final state of the collapsed particle. The number of consecutive images taken before reaching each disassembly milestone is indicated for each panel. Reproduced from Ref. [75••].

Figure 2



Architecture of the adenovirus icosahedral shell.
(a) General view of the HAdV-C5 molecular model reported in Ref. [27]. The bar represents 10 nm. The color legend at the right hand side applies to the proteins depicted in panels (a), (b) and (c). Numbers in parenthesis indicate the copy number of each protein in the virion. The fibers are not modeled. **(b)** A view from inside the capsid showing the traced internal proteins. Only a very small fragment of core protein VII is observed. The view is rotated by 180°C around a vertical axis with respect to (a). **(c)** Zoom in on the icosahedral facet in the same orientation as in (a), with all proteins except pentons and IX removed, to highlight the intricate network established by protein IX on the outer capsid surface. **(d)** Comparison of the decorating proteins IX and LH3 in the structures of mastadenoviruses (HAdV-C5, PDB entry 6B1T) and atadenoviruses (LAdV-2, PDB 6QI5). The triskelion domain, formed by trimerization of the protein N-terminal domains, is very similar in both proteins, while the fold of the rest of the molecule is completely different.

to the capsid surface. This is characteristic of the adenovirus structural lineage, nowadays filed under the *Bamfordvirae* kingdom, *Varidnaviria* realm in the taxonomic classification of viruses [33]. A single jelly roll protein, penton base, forms the vertex pentamers. Trimeric fibers of different length and flexibility depending on the particular virus type protrude from the vertices [5]. Minor coat proteins IIIa, VI and VIII on the inner capsid surface are common to all *Adenoviridae* family members (Figure 2b), while external decorating proteins vary between genera [34]. Atadenoviruses display on their surface a protein with a trimeric β-helix fold called LH3 [35]; the external cementing protein in mastadenoviruses is called IX and forms trimeric or tetrameric α-helical bundles (Figure 2c) [12,29,36], except in enteric adenoviruses where most of the protein seems to be disordered [30•,31•]. No external decorating protein has been found for the other adenovirus genera. LH3 and IX attach to the capsid surface via the same triskelion-shaped structural feature, indicating a common origin and divergence to very different architectures throughout evolution (Figure 2d). The β-helix domain of LH3 was probably captured from a bacterial gene, incorporated as capsid decoration in atadenoviruses, and later on duplicated to become protein IX and the non-structural, oncoprotein E1B 55 K in mastadenoviruses [28••]. Although part of the conserved capsid proteins, IIIa presents different conformations in the two genera studied so far (mastadenoviruses and atadenoviruses), altering the network of interactions on the inner capsid surface [28••].

Adenovirus genome: packing and packaging

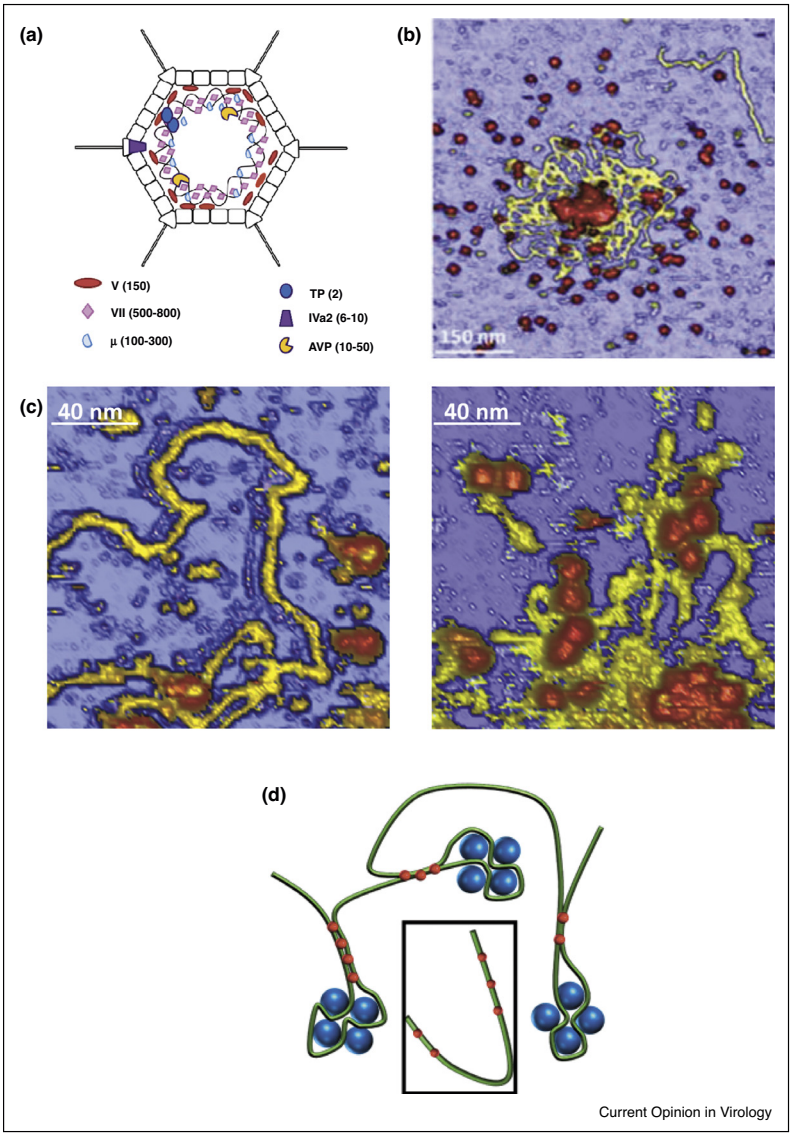
The adenovirus capsid encloses the genome (36 kbp in HAdV-C5) packed together with positively charged, virus-encoded proteins: core polypeptides V (present only in mastadenoviruses), VII and μ. Proteins involved in genome replication (terminal protein, TP) and encapsidation (IVa2), as well as the adenovirus maturation protease (AVP) are also bound to the genome in the virion (Figure 3a). These proteins account for ∼20 MDa, approximately 50% of the total core mass. Cryo-EM single particle averaging yields featureless density for the core, indicating lack of icosahedral symmetry, and maybe even a different organization in each virion. Structural data on core proteins are limited to a 10-residue peptide in protein VII, which was located inside the hexon trimer central cavity in the latest HAdV-C5 structure (Figure 2b) [27]. Further, estimated copy numbers vary depending on the methodology used [37–40] (Figure 3a). Lack of basic data together with high crowding also hinders interpretation of cryo-electron tomography data. Statistical analyses of density maxima distribution in the core of HAdV-C5 tomography maps yielded a model for the interactions present inside the virion, which would be similar to those in a fluid of soft repulsive particles [41]. This soft electrostatic repulsion is consistent with the expected screening of ∼45% dsDNA negative charges by the core proteins positive charges [42], a phenomenon thought to be crucial for genome packing within the confined capsid space.

The observation that adenovirus particles can be assembled in the absence of the most abundant core protein (polypeptide VII) raised questions regarding the actual role of these 'histone-like' proteins [43]. Virus particles can be cracked open by a single AFM nanoindentation [24], or gradually disrupted by mechanical fatigue induced by imaging at low forces [44]. Using these approaches on wildtype HAdV-C5 particles (Ad5-wt) and particles lacking protein VII (Ad5-VII-) allowed a direct comparison of genome release timing and topology of extracted contents [45•]. These experiments demonstrated that Ad5-VII- particles release their dsDNA earlier than Ad5-wt, consistent with lower genome condensation in the absence of protein VII. AFM images of core components expelled after mechanical disruption of the virion (Figure 3b) showed particles compatible with ∼9 copies of VII, interspersed with bundles of DNA fibers with a thickness up to 3 times that of a dsDNA helix (Figure 3c). These data prompted a model where protein VII condenses the adenovirus genome by combining direct clustering and promotion of bridging by other core proteins (Figure 3d) [45•].

How the adenovirus genome and its accompanying proteins enter the capsid is poorly understood. A specific sequence located at the genome left end, plus five virus-encoded proteins (IVa2, L1 52/55 kDa, L4 22 kDa, L4 33 kDa, and IIIa) are required for genome packaging to occur [46]. Low resolution (∼12 Å) cryo-EM studies on genome-less capsids stalled at different maturation stages showed that L1 52/55 kDa forms a disordered shell with preferential location under the vertices, in agreement with its interaction with protein IIIa, which forms part of the icosahedral capsid [12,47]. This interaction defines packaging specificity [48].

Because one of the packaging proteins (IVa2) is a putative ATPase, adenovirus has a dsDNA genome, and produces large amounts of empty capsids, it had been assumed that its assembly and packaging would occur sequentially, by genome translocation through a portal similar to that of tailed phages [46]. However, no clear NTPase activity has been found for IVa2 [49,50]; molecular characterization of incomplete particles suggests that empty capsids are not assembly intermediates but abortive products [47]; and association of the adenovirus genome with core proteins poses a considerable topological complication for DNA translocation. Conventional EM images of cells infected with a delayed packaging HAdV-C5 mutant showed images consistent with partially formed capsids engulfing the viral DNA (Figure 1a), suggesting that assembly and packaging occur in a concurrent, rather than sequential, manner [51]. The role of the packaging proteins would be

Figure 3



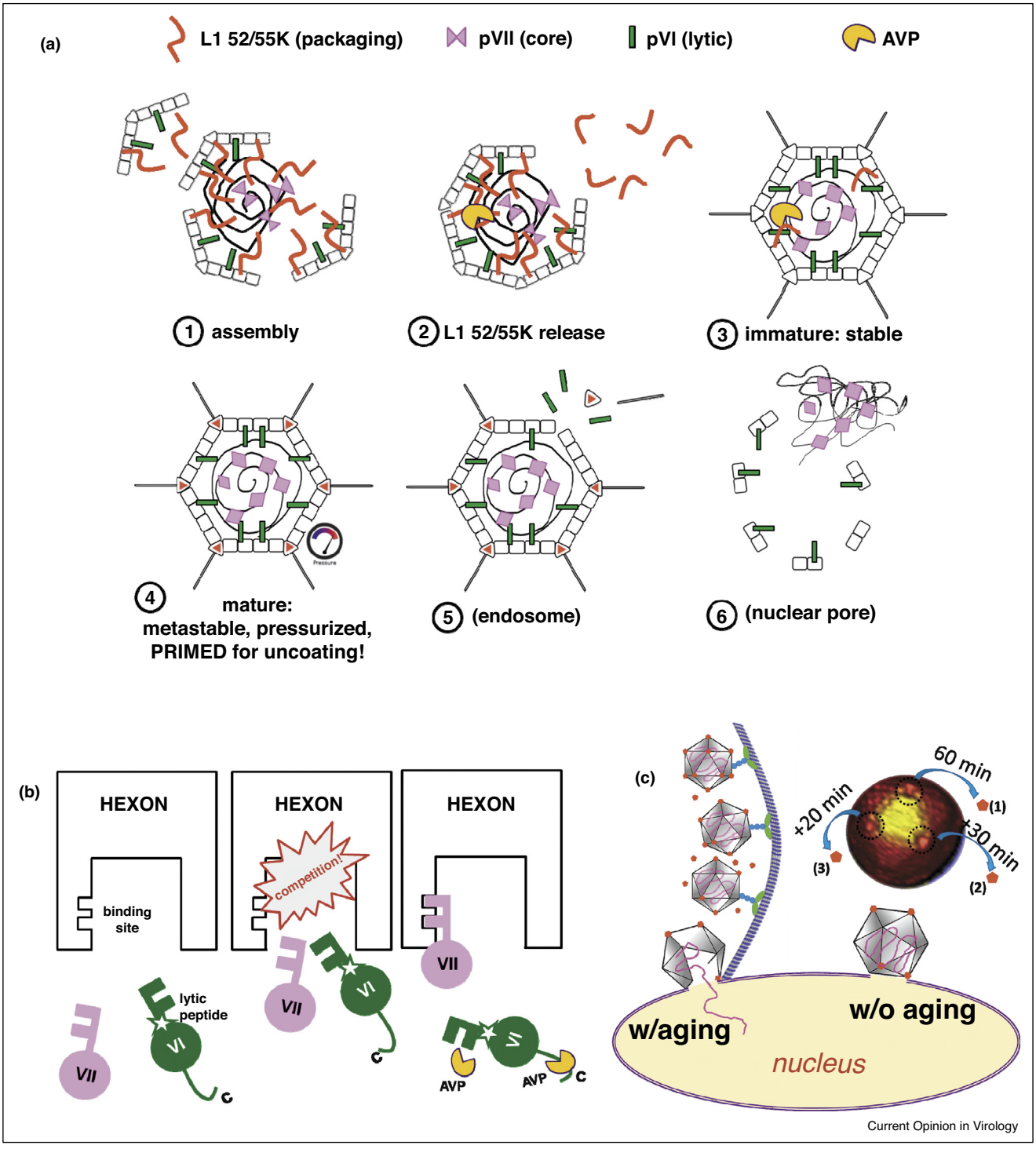
Organization of the adenovirus core.
(a) Schematics showing the core components. Numbers in parenthesis indicate the estimated copy number for each protein. TP is the terminal protein bound to the 5′ ends of the genome [82]. Polypeptide IVa2, the putative packaging ATPase, has been reported to occupy a singular vertex in the capsid [83]. Modified from Ref. [11]. **(b)** Mechanical fatigue disrupted adenovirus particle showing DNA release. **(c)** AFM images showing released core components in Ad5-VII- (left) and Ad5-wt (right) particles after mechanical fatigue disruption. Fibered shapes corresponding to dsDNA (yellow) appear as thick bundles in Ad5-wt and reflect the condensing action of protein VII. Homogeneously dispersed formations (blobs, red) were interpreted as clusters formed by dsDNA and oligomers of protein VII. **(d)** Cartoon showing a hypothetical model for the condensing role of protein VII. Green, DNA; blue, protein VII; red, protein μ. The inset illustrates the situation where the condensing action of μ is hindered by the absence of clusters formed by the VII-DNA complexes. Panels (b), (c) and (d) reproduced from Ref. [45•].

to recruit capsid subassemblies to genomes, rather than forming a portal, and modulate the capsid-core interactions during assembly (Figure 4a, **step 1**) [51].

What about genome UNpacking?

Production of mature, infective adenovirus virions requires proteolytic processing by AVP of proteins IIIa, VI and VIII on the inner capsid surface; VII and μ in the core; and packaging protein L1 52/55 kDa [52,53]. Maturation is linked to successful virion uncoating, which starts upon attachment to cell receptors and continues in early endosomes. A key step in adenovirus entry is exposure of the membrane-lytic peptide present in protein VI, to facilitate membrane penetration and escape

Figure 4



Adenovirus assembly and disassembly as revealed by combined EM-AFM studies.
(a) Cartoon summarizing the adenovirus assembly and maturation pathway and its effects in priming for uncoating (see main text for details). Modified from Ref. [84•] **(b)** Model for competition between proteins VI and VII during HAdV-C5 assembly. The N-terminal regions of both proteins (keys) compete for the same binding pocket in the hexon monomer (lock). This competition forces one of the two binding candidates out of the hexon cavity, making it accessible to AVP cleavage, and in the case of protein VI, for exposure and membrane disruption in the endosome. Modified from Ref. [73••]. **(c)** Penton aging revealed by analysis of HAdV-C5 mechanical fatigue disruption at different forces. Aging guarantees that the particle is ready to liberate the genome at the nuclear pore, after initial disruption during endocytosis and transport along the microtubular network. Modified from Ref. [75••].

to the cytosol [53,54•,55,56]. Partially disrupted particles must be stable enough to endure transport along microtubules and reach the nuclear pore, where they are dismantled and the viral DNA, together with core protein VII, enter the nucleus [57,58]. Immature particles are defective in the initial stages of uncoating: they do not release fibers upon receptor binding, nor the lytic peptide in early endosomes. Consequently, they become trapped in the endocytic pathway, and infection is aborted [54•,55,59–62].

Unlike other dsDNA viruses, such as herpesvirus and many tailed phages, adenovirus capsids do not undergo large conformational rearrangements upon maturation [63–66]. Combined cryo-EM (single particle averaging or electron tomography) and AFM analyses have shown that maturation disrupts interactions in both capsid and core that stabilize the immature particle (Figure 4a, **step 3**). Maturation primes the virion for uncoating (Figure 4a, **4**) by facilitating vertex release (Figure 4a, **5**) and loosening the condensed genome and its attachment to the icosahedral shell (Figures 4a, **6** and 1c) [44,47,52,63,67–70]. Interaction with host factors during entry, in particular receptors (CAR/integrins) also destabilize pentons [71,72]. Penton loss is a requisite for exposure of the lytic peptide and subsequent endosomal escape (Figure 4a, **5**), while genome detachment from capsid fragments must happen at the nuclear pore (Figure 4a, **6**). AFM probing of the mechanical properties of adenovirus mature and immature capsids and cores revealed that maturation increases the internal pressure exerted by the genome, providing a physical mechanism to facilitate penton release (Figure 4a, **4**). This pressure is originated by electrostatic repulsion between confined DNA strands, and its increase seems to be due to a reduction of the DNA condensing action of VII and μ upon cleavage by AVP [69].

Packaging protein L1 52/55k is also a substrate for AVP [52]. L1 52/55k can form homooligomers and interact with both capsid and core components, acting as a 'Velcro' tether during assembly (Figure 4a, **1**). Proteolytic cleavages disrupt interactions between L1 52/55k and other virion components, facilitating its removal during maturation (Figure 4a, **2**) and separation of the genome from capsid fragments in the final stages of uncoating (Figure 4a, **6**) [47,51,52].

Protein VII is not required for genome packaging . However, Ad5-VII- particles are deficient in maturation of protein VI, become trapped in endosomes and are therefore non-infectious [43]. Only two disconnected regions of protein VI have been identified in the virion structure, and intriguingly, a small peptide of core protein VII was also modeled binding to the same hexon region as protein VI (Figure 2b) [27]. Combined fluorescence, AFM and cryo-EM studies showed that Ad5-VII- particles become trapped in endosomes because they fail to expose protein VI during entry, but lack of VI exposure is not due to increased particle stability [73••]. Quite the contrary, penton release and capsid disruption happen at lower thermal or mechanical stress in Ad5-VII- than in Ad5-wt particles. Comparison of Ad5-wt and Ad5-VII- cryo-EM maps confirmed that the precursors of protein VII and VI compete for the same binding pocket in hexon, and showed density consistent with the lytic peptide of VI trapped inside the hexon cavity in the absence of protein VII [73••]. These observations support a model where competition between proteins VI and VII for hexon binding during assembly would be responsible for releasing the lytic peptide from the hexon cavity, facilitating its exposure during uncoating in the endosome (Figure 4b). This competition would also ensure that the VI and VII precursors leave the protection of the hexon cavity to become accessible to AVP for maturation cleavages, as the protease uses the viral genome as a sliding track to reach all its substrates inside the particle [53]. The behavior of Ad5-VII- particles illustrates the result of displacing the competition between VI and VII towards a VI-win. A protein VI mutant (S28C) with a change near the residue filling the common binding pocket in hexon (Ser31) represents the opposite effect: S28C has correct processing of VI, in contrast to Ad5-VII-, but deficient maturation of VII. S28C particles present increased stiffness, presumably caused by enhanced bridging between shell and genome by the uncleaved pVII [74].

Mechanical fatigue induced by repeated AFM scanning of the same particle at low force (∼100 pN) induces stepwise disassembly of adenovirus particles, mimicking the process occurring in the cell [44,59,68] (Figure 1i). This methodology showed that penton release occurs in a sequential manner, and mature adenovirus particles are prone to loose pentons twice faster than the immature ones [44], opening the way for lytic peptide exposure. Additionally, viral genomes escape faster from crack-opened mature than immature virus particles [68].

Controlled uncoating is a critical part of the infectious cycle, as virions must fall apart in an orderly fashion to release their genomes at the appropriate time and place for successful replication and transcription. *In vitro*, adenovirus DNA escapes through fractures that only happen after several pentons are lost (Figure 1i) [44,45•,75••]. Survival analysis applied to observations from mechanical fatigue experiments revealed that the probability of a penton to be released increases if other pentons have already been removed. This aging effect accelerates penton disruption by 50% over a process without aging (Figure 4c) [75••]. Pentons act as a 'lock' of the virus structure [76], which turns more unstable as more and more pentons are lost. It is likely that particles retaining too many pentons would not be able to release their DNA

at the nuclear pore, whereas those with too few pentons might lose their genome before reaching the nucleus, or expose it to the cell defense machinery (Figure 4c). Aging would tune the sequential loss of pentons to render a subviral particle stable enough to withstand travel along the microtubule network, but ready to crack-open for genome delivery at the nuclear pore.

The study of Ad5-VII- particles indicated that, although protein VII is dispensable for the genome packaging [43], it has important implications in adenovirus biomechanics and uncoating. Absence of VII results in an excess internal pressure, pentons are more easily released and DNA diffuses faster out of the capsid [45•,73••]. Thus, the condensing action of protein VII modulates internal capsid pressure and genome release, which could be crucial to keep the genome protected while the partially uncoated particle travels to the nuclear pore.

We have discussed two examples where the condensation state of the adenovirus core is affected by the presence, or maturation state, of the DNA condensing proteins. Most recently, a change in core condensation upon chemical changes in the environment has also been described. A shift from neutral (pH 7.8) to acidic (pH 6-4) conditions decreased the stiffness of HAdV-C5 virions from 0.58 N/m to 0.44 N/m. The stiffness of empty capsids remained unaltered at low pH, suggesting that the mechanical change was related to changes in the core. In extrinsic fluorescence thermostability assays, acidification made the genome less accessible to an intercalating dye, even in the presence of capsid openings revealed by conventional electron microscopy. All these observations, together with estimations of the DNA/protein electrostatic charge balance, prompted the conclusion that acidic pH condenses the core by increasing the proportion of DNA negative charges screened by the core proteins [42]. This extra condensation could help keep the core integrity during the initial uncoating events. However, the actual effect of acidification in adenovirus stability, and its biological relevance in entry, are unclear: *in vitro* disruption assays carried out at acidic pH have provided conflicting results [42], and in-cell assays have shown that endosome escape does not require acidic pH [77].

Conclusions

Although adenoviruses have been studied for over 60 years, many aspects of their infectious cycle remain unclear. Cryo-EM high resolution structures have considerably improved our knowledge on the organization of the icosahedral shell [5]. Thanks to the combination of structural and physical analyses, a model for adenovirus assembly and disassembly is emerging in which core proteins are not mere 'subway pushers', helping to squeeze the genome inside the capsid by screening its negative charges. The presence and maturation state of core proteins regulate genome condensation, penton release, genome diffusion out of the capsid and exposure of the membrane-lytic peptide during entry. *In vitro*, pentons are released in a sequential and highly cooperative way, finely tuned to produce a particle prepared for genome release at the nuclear pore.

Fundamental questions such as the relationship between capsid architecture, particle stability and specific tropisms still remain to be answered. For example, it is still not clear what role acidification plays in adenovirus uncoating in the cell [42], how the different decorating proteins may influence host specificity [28••,29,30•,31•], or what are the functions of core proteins V and μ. Structural methods are nowadays revealing fundamental non-icosahedral elements in viral capsids [78], but have only started to be used in adenovirus analyses [79]. The dynamics of genome release have been difficult to tackle, as only material adsorbed to the substrate can be imaged by AFM. AFM-TIRF combination appears as a promising tool to address this subject *in vitro* by allowing genome exposure evaluation by two complementary techniques [68]. Finally, studies carried out on isolated particles may soon be complemented by observing adenovirus assembly and uncoating in the cellular context, using FIB-SEM and cryo-electron tomography approaches that have already shown unprecedented images on labile, impossible to purify assembly intermediates in other viruses [80].

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Economy, Industry and Competitiveness (FIS2017-89549-R; 'Maria de Maeztu' Program for Units of Excellence in R&D MDM-2014-0377; and FIS2017-90701-REDT) and from the Human Frontiers Science Program (HFSPO RGP0012/2018) to P.J.P.; as well as grant PID2019-104098GB-I00/AEI/10.13039/501100011033, co-funded by the Spanish State Research Agency and the European Regional Development Fund to C.S.M. The CNB-CSIC is further supported by a Spanish State Research Agency Severo Ochoa Excellence grant (SEV 2017-0712). C.S.M. is a member of the CSIC funded consortium LifeHub (CSIC grant number: 202120E47). REACT-EU funding by Comunidad Autónoma de Madrid is also acknowledged. The authors thank all their past and present group members, collaborators and colleagues who have contributed to the work reviewed here.

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- • of special interest
- •• of outstanding interest

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